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Biochimica et Biophysica Acta 1592 (2002) 163–167
BIOCHIMICA ET BIOPHYSICA ACTA
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Regulation of Egr-1 by association with the proteasome component C8

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Received 14 March 2002; received in revised form 8 August 2002; accepted 8 August 2002

Abstract

Primary response transcription factor, Egr-1, is rapidly activated by a variety of extracellular stimuli. Activation of Egr-1 is shown to function as a master switch activated by ischemia to trigger expression of pivotal regulators of inflammation, coagulation and vascular hyperpermeability. Egr-1 is a short-lived protein, but the mechanism that regulates its stability has not yet been clarified. In this study, the yeast two-hybrid screening revealed that Egr-1 interacts significantly with PRC8 (proteasome component C8) and the specific interaction was confirmed by GST pull-down assay and coimmunoprecipitation. Interestingly, we found that the PRC8-mediated regulation of Egr-1 activity is associated with the proteasome pathway and PRC8 inhibits the transcriptional activity of Egr-1. In addition, Egr-1 protein was specifically multiubiquitinated by ubiquitin. These data strongly imply that Egr-1 protein is targeted for proteolysis by the ubiquitin-dependent proteasome pathway.

Keywords: Egr-1; Proteasome; Ubiquitin; Protein degradation

1. Introduction

Egr-1 is a zinc finger transcription factor and one of immediate early response genes in a multigene family that includes Egr-2, Egr-3, Egr-4 and WT1 [1,2]. Egr-1 is an 80–82 kDa protein consisting of 533 amino acids, discovered independently by a number of laboratories searching for factors regulating cell growth and proliferation [1,3]. In addition, Egr-1 is upregulated in response to a wide variety of mitogenic and nonmitogenic stimuli, including peptide growth factors, shear stress, urea, hypotonicity and hypoxia [4–9]. It is thus likely that Egr-1 protein levels are critically regulated during development, differentiation and tumorigenesis.

Once activated, Egr-1 binds to 5′-GCG(G/T)GGGCG-3′ consensus sequences within the promoter region of target genes, resulting in transcriptional activation or repression. Egr-1 bound to DNA alters gene transcription through mechanisms dependent on both coactivators and corepressors. Transcriptional coactivators, such as CBP and p300, can interact with the activation region of Egr-1 and increase the transcriptional activity of Egr-1 [10]. Corepressors such as NAB1 and NAB2 also interact with Egr-1 and negatively regulate Egr-1 activity [11,12].

In view of protein stability, Egr-1 is a short-lived protein with a half-life of less than 2 h [2]. However, the mechanism underlying the regulation of the stability of the Egr-1 protein has not yet been elucidated. Recently, it has been shown that the degradation of various transcription factors, such as c-Jun [13], c-Fos [14], STAT1 [15], p53 [16] and c-Myc [17], is mediated by the ubiquitin–proteasome pathway, which is a major route of intracellular degradation of short-lived regulatory proteins [18].

In this paper, we demonstrate that PRC8, α-type subunit of 20S proteasome core complex, interacts with Egr-1, leading to the ubiquitin-dependent proteasome degradation of Egr-1.

Abbreviations: ALLM, *N*-Acetyl-Leu-Leu-Met-al; ALLnL, *N*-Acetyl-Leu-Leu-norLeu-al; Egr-1, early growth response-1; PRC8, proteasome component C8; Ub, ubiquitin

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PII: S0167-4889(02)00310-5

2. Materials and methods

2.1. Materials

E64 and ALLM were purchased from Sigma. ALLnL, MG132 and lactacystin, were purchased from Calbiochem. Egr-1, α-tubulin and FLAG antibodies were purchased from Santa Cruz Biotechnology, InnoGenex and Sigma, respectively.

2.2. Plasmids

For the yeast two-hybrid screening, we amplified the C-terminal region of Egr-1 spanning amino acids 420–543 by PCR and subcloned into the pBHA. To construct PRC8 expression vector, PRC8 cDNA was inserted into the pCMV-Tag2 (Stratagene). Egr-1 expression vector was also constructed by PCR and subcloned into the pCMV-Tag2 (Stratagene). FLAG-Ub vector was kindly provided from Dr. H. Yokosawa (Hokkaido University, Sapporo, Japan).

2.3. Two-hybrid library screening and evaluation of protein–protein interactions

Yeast strains SFY526 and L40 obtained from CLONTECH were used to assay protein–protein interactions and for library screening, respectively. Two-hybrid assays using the LexA system were performed according to the instructions of the manufacturer (CLONTECH).

2.4. GST pull-down assays

[^{35}S]Methioine-labeled in vitro translated Egr-1 was prepared by using the TNT system (Promega). GST fusion proteins were purified as described previously [19]. Equal amounts (~ 1 μg) of GST or GST-PRC8 immobilized on glutathione sepharose beads was incubated with [^{35}S]Egr-1 in reaction buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40) at 4 °C. After washing, the bound proteins were eluted with the sample buffer and were separated by SDS-PAGE, followed by autoradiography.

2.5. Coimmunoprecipitation

After 24 h of transfection into HEK 293 cells, the cells were washed with PBS. The preparation of protein extracts, immunoprecipitation and Western blot were performed as described previously [20].

2.6. Luciferase assay

The human IGF-II P3 construct, Hup3 [21], was used as luciferase reporter plasmid. The 0.4 μg each of Hup3 and pCMV/β-gal with or without 1 μg of Egr-1/PRC8 were transfected to HEK 293 cells. Luciferase assay was performed as described previously [22].

2.7. Ubiquitination assay

After transfection of pCMV-Egr-1 and FLAG-Ub to HEK 293 cells, 24 h later, the cells were treated with 5 μM of MG132 for 12 h. Cell lysates were immunoprecipitated with Egr-1 antibody. Precipitated proteins were separated by SDS-PAGE and immunoblotted with FLAG antibody.

3. Results

3.1. Egr-1 interacts with the PRC8 through the C-terminal region

The yeast two-hybrid system was used to identify candidate proteins that interact with Egr-1. As shown in Fig. 1A, mouse embryonic 17-day cDNA library was screened with the C-terminal region of Egr-1 as a bait. From 6×10^6 transformants, 92 His^+/Lac^+ double-positive clones were isolated. The 16 clones were further selected by β-galactosidase assays in another strain, SFY526. DNA sequence and

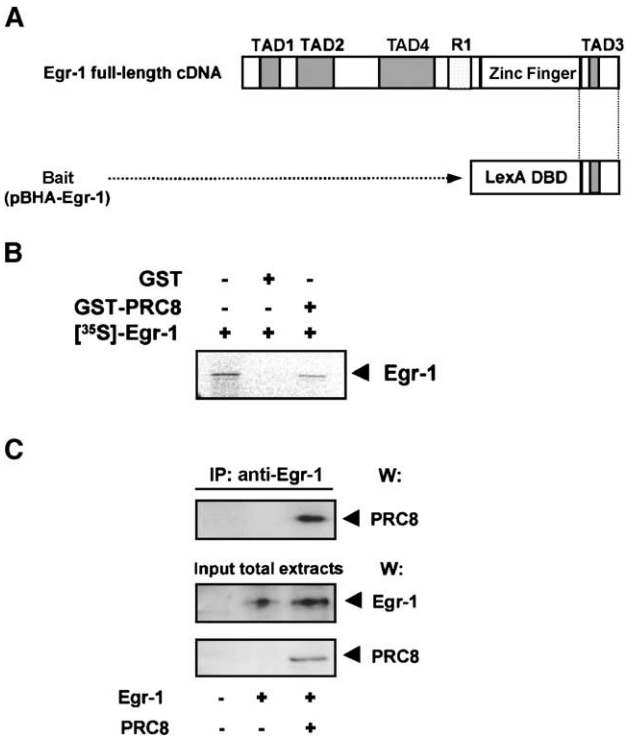

Fig. 1. Association of Egr-1 with PRC8. (A) Structures of Egr-1 proteins tested in yeast two-hybrid screening are shown. TAD, transactivation domain; DBD, DNA binding domain; R1, repression domain. (B) In vitro translated Egr-1 was incubated with GST-PRC8, washed, and separated by SDS-PAGE. (C) pCMV-Egr-1 was transfected into HEK293 cells together with pCMV-PRC8. Cells were lysed and immunoprecipitated with Egr-1 antibody. Immunopurified proteins were analyzed by Western blot with FLAG antibody. W, Western blot.

database searches revealed that the nucleotide sequence of 12 clones encoded mouse PRC8.

3.2. Egr-1 interacts with PRC8

To confirm that PRC8 interacts with Egr-1, we performed GST pull-down assays. As expected, Egr-1 was pulled down with GST-PRC8 but not with GST alone (Fig. 1B), confirming that the interaction between PRC8 and Egr-1 is specific in vitro. To further verify this interaction, we performed coimmunoprecipitation assay. As shown in Fig. 1C, Egr-1 strongly interacts with PRC8.

3.3. Association of Egr-1 with PRC8 causes its degradation through the proteasome pathway

Association of Egr-1 with PRC8 raised the possibility that Egr-1 could be degraded through a PRC8-dependent proteasome degradation pathway. To investigate whether this pathway plays an important role in the degradation of Egr-1, we first examined the effects of proteasome and protease inhibitors on the steady-state level of Egr-1 protein (Fig. 2A). Treatment with the proteasome-specific inhibitors, ALLnL, MG132 and lactacystin on HepG2 cells, resulted in elevation of Egr-1 protein levels remarkably, but not with nonproteasome inhibitors such as E64 and ALLM. To study whether the above effects are specific at the protein level, we also examined the effects of the proteasome inhibitors on the level of Egr-1 mRNA, and found that any of the inhibitors did not change the expression of Egr-1 mRNA (Fig. 2B). Next, to determine whether

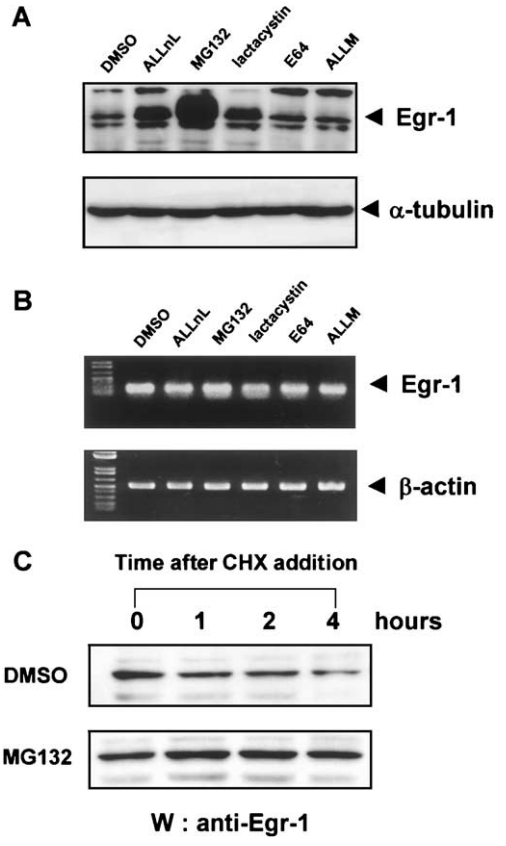

Fig. 2. Effects of proteosome-specific inhibitors on the levels of Egr-1 protein and mRNA. (A) HepG2 cells were treated with proteosome and protease inhibitors for 4 h. The total proteins were analyzed by Western blotting with Egr-1 and α-tubulin antibodies, respectively. (B) Total RNA was extracted and the levels of Egr-1 and β-actin mRNA were analyzed by RT-PCR. (C) Effect of proteasome-specific inhibitor on the stability of Egr-1 protein. HepG2 cells were treated with 50 μM MG132 or 0.25% DMSO for 1 h followed by incubation with cycloheximide (CHX) at a concentration of 25 μg/ml for the indicated periods. The cell lysates were prepared and the protein levels of Egr-1 were analyzed by Western blotting with Egr-1 antibody.

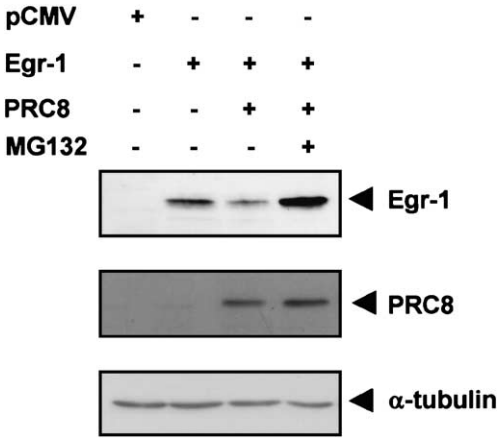

Fig. 3. Destabilization of Egr-1 by PRC8. pCMV-Egr-1 was cotransfected with or without pCMV-PRC8, as indicated. Total cell lysates were subjected to Western blotting. Egr-1 and PRC8 were detected with FLAG antibody.

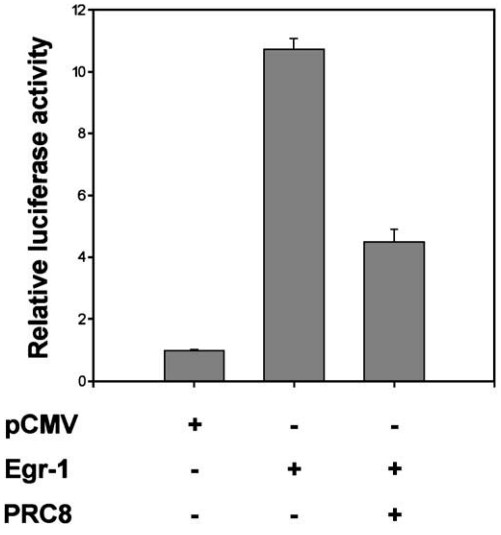

Fig. 4. PRC8 inhibits the transcriptional activity of Egr-1. HEK293 cells were transfected with 0.4 μg each of the reporter plasmid Hup3 and 1 μg of pCMV-Egr-1, and 0.5 μg of pCMV/β-gal together with 1 μg of pCMV-PRC8. Luciferase activities were normalized to β-galactosidase activities. This is a representative of three independent experiments that were done in duplicate.

the proteasome inhibitor directly affects degradation of Egr-1 protein, we measured its effects on the stability of Egr-1 protein under conditions in which protein synthesis had been blocked by cycloheximide. As shown in Fig. 2C, Egr-1 protein was stabilized in the presence of MG132, indicating that the proteasome inhibitor directly inhibited degradation of the Egr-1 protein. These results demonstrate that Egr-1 protein is degraded by the 26S proteasome.

3.4. PRC8 destabilizes Egr-1

PRC8 is a subunit of the 26S proteasome complex that degrades poly-ubiquitinated proteins. Since our results provided evidence that PRC8 interacts with Egr-1 and the proteasome-specific inhibitors increased the stability of Egr-1 protein, we examined whether PRC8 affect the stability of Egr-1 protein through the proteasome-dependent pathway. Interestingly, coexpression of PRC8 decreased the Egr-1 protein level (Fig. 3), suggesting that PRC8 destabilizes Egr-1.

3.5. Effects of PRC8 on the transcriptional activity of Egr-1

We then performed luciferase reporter assays to test whether PRC8 regulates the transactivation activity of Egr-1 through the regulation of the stability of Egr-1. As shown in Fig. 4, transfection of pCMV-Egr-1 with the reporter plasmid resulted in an increase of the luciferase activity. In contrast, cotransfection of pCMV-PRC8 decreased the transactivation activity of Egr-1, indicating that PRC8 inhibits the transcriptional activity of Egr-1.

3.6. Egr-1 is multiubiquitinated

We next investigated whether Egr-1 could be ubiquitinated or not. Transiently expressed Egr-1 protein produced high molecular mass bands even under SDS-PAGE and the presence of these bands suggested the ability of Egr-1 to produce a variety of stable protein complexes including Ub–Egr-1 complexes that was dependent on MG132 concentration (data not shown). To confirm whether Egr-1 protein is multiubiquitinated before degradation by 26S proteasome, we transiently overexpressed both FLAG-Ub and Egr-1 in HEK293 cells simultaneously. High molecular mass materials, detected by FLAG antibody, accumulated extremely in the case of cotransfection with Ub and Egr-1 expression plasmids (Fig. 5). Altogether, these results strongly suggest that Egr-1 protein is multiubiquitinated before its degradation by the 26S proteasome.

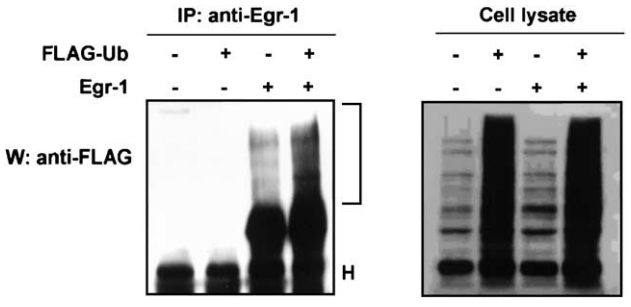

Fig. 5. Ubiquitination of Egr-1. HEK293 cells were transiently transfected with the indicated combinations of FLAG-Ub and pCMV-Egr-1, and 24 h after transfection, the cells were treated with 5 μM MG132 for 12 h. The bracket indicates multiubiquitinated Egr-1. Cell lysates were directly subjected to Western blotting with FLAG antibody to check the expression level of FLAG-Ub. H, IgG heavy chain.

4. Discussion

Expression of Egr-1 is probably closely regulated under normal conditions. Overexpression of Egr-1 has been shown in Burkitt lymphoma [23]. In contrast, loss of Egr-1 expression has been shown to confer an immortalized phenotype in several murine and human cell lines [23]. Moreover, low expression of Egr-1 has been demonstrated in human lung cancer compared with normal lung tissue [24]. However, our previous results indicated that Egr-1 is directly involved in the expression of IGF-II [8], which is expressed in most of the cirrhotic and human hepatocellular carcinoma tissues [25,26] and has an angiogenic activity [27,28]. Thus, altered gene expression of Egr-1 may be an important step in the progression of cells from normal to the malignant phenotype. However, posttranslational regulation mechanism of Egr-1 protein is not clear.

The selective degradation of many short-lived proteins in eukaryotic cells is carried out by the ubiquitin-dependent 26S proteasome [29]. The 26S proteasome is a 2.5-MDa molecular machine built from ~31 different subunits, which catalyzes protein degradation [29]. The 26S proteasome complex is a ubiquitous multicatalytic protease complex composed of two large complexes: the 20S catalytic core complex and the 19S regulatory complex [30]. The 19S complex is required for the recognition of poly-ubiquitinated protein substrates that are degraded inside of the 20S core complex. The barrel-shaped 20S particle is made up of four rings, each of which contains seven different subunits. The two inner rings contain β-type subunits and the outer rings comprise α-type subunits. The proteasome is involved in many different cellular processes, ranging from the cell cycle process to antigen processing [29]. Recently, there have been several reports about cellular regulatory proteins and viral proteins that interact with subunits of the proteasome complex and participate in the proteasome-dependent regulation [31–33].

In our work, the specificity of PRC8 interaction with Egr-1 was markedly demonstrated in a yeast two-hybrid assay and was further confirmed in vitro (Fig. 1). These results indicated that PRC8 is involved in the regulation of Egr-1. Significantly, PRC8-mediated regulation of Egr-1 activity is associated with the proteasome pathway (Figs. 2 and 3) and we showed that PRC8 decreased the transcrip-

tional activity of Egr-1 (Fig. 4). These negative regulation of Egr-1 by PRC8 may be due to the PRC8-mediated recruitment of Egr-1 to the proteasome complex. We further demonstrated that Egr-1 is multiubiquitinated (Fig. 5). Thereby, PRC8 might be required for rapid degradation of the Egr-1 protein through the ubiquitin–proteasome system.

Our finding is consistent with previous reports that various short-lived transcription factors are degraded by ubiquitin–proteasome pathway [13–17]. Especially, this is the first report of Egr-1 protein degradation mechanism through the ubiquitin–proteasome pathway. Taken together, these results strongly imply that Egr-1 protein is targeted for proteolysis by the ubiquitin-dependent proteasome pathway through association with PRC8. In addition, it can be suggested that the action of Egr-1 on cell cycle progression, development and tumorigenesis is controlled through degradation by the ubiquitin–proteasome pathway.

Acknowledgements

Financial support was from the National Research Laboratory Fund (2000-N-NL-01-C-015), the Ministry of Science and Technology, Korea (to K-W. K).

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